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GENETIC CONTROL OF FLOWERING TIME IN *EUCALYPTUS GLOBULUS* SSP. *GLOBULUS*

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ABSTRACT

Understanding the factors affecting variation in phenology within a species is important as flowering time constitutes one of the major barriers to gene flow. We studied the genetic and environmental control of flower initiation and anthesis time in *E. globulus* ssp. *globulus*. For five years, flower initiation and anthesis were monitored in a seed orchard containing clones of 63 genotypes from four different regions of the species' natural distribution. Anthesis occurred over a long period each year, spanning as much as nine months in 2008. This variation was under strong genetic control with little genotype by year interaction (broad-sense heritability, $\hat{H}^2$=0.78±0.04). There were highly significant differences among regions; anthesis occurred earlier for Furneaux and Tasmania than Strzelecki and Otways each year. Surprisingly though, there was little variation in flower initiation time between regions and genotypes, and this was under weak genetic control ($\hat{H}^2$=0.06±0.05). The average anthesis time in the orchard varied from year to year and there was evidence that heat sum was a major driver of this environmental variation. Anthesis time is controlled by both genetic and environmental factors, with the responses to each being predictable to some extent, and unrelated to the timing of flower initiation.

Key words: flowering time, flower initiation, anthesis, genetic control, phenology, heat sum, seed orchard.

INTRODUCTION

Eucalyptus globulus ssp. *globulus* (blue gum) is one of the most widely planted eucalypts in temperate parts of the world, including its own natural range in south-eastern Australia (Barbour et al. 2008; Eldridge et al. 1993). Understanding the genetic and environmental control of flowering in *E. globulus* ssp. *globulus* is important as this trait is a major determinant of gene flow between natural populations of plants (Levin 1978), influences the tendency of co-occurring eucalypt species to hybridise (Griffin et al. 1988), and affects the potential for gene flow between plantations and native forest (Barbour et al. 2008). In addition, an understanding of the timing of floral initiation and flower opening (anthesis) among elite genotypes is required for successful management of open pollinated seed orchards, which are used to produce genetically improved *E. globulus* ssp. *globulus* seed for plantations (Potts et al. 2008).

The *E. globulus* species complex is naturally distributed in south-eastern mainland Australia and on the island of Tasmania. It consists of four taxa variously described as species or subspecies (*bicostata*, *globulus*, *maidenii* and *pseudoglobulus*) but most recently as subspecies (Slee et al. 2006). Core populations of these taxa are morphologically and geographically distinct, but they are linked by intergrade populations that are intermediate in morphology (Jordan et al. 1993). The populations used in plantations are not only from core *E. globulus* ssp. *globulus* but also include some intergrade populations. Nevertheless, here we will refer to all populations as *E. globulus*.

E. globulus has a long juvenile and non-flowering phase of one to five years (Jordan et al. 2000), followed by an adult phase consisting of an annual cycle of vegetative and reproductive growth. It is evergreen, but over the winter months in south-eastern Australia, vegetative growth is limited. When in adult phase, and following the annual spring flush of vegetative growth, floral buds are observed in leaf axils, though the flower buds probably initiate at the microscopic level several weeks before they are visible to the naked eye (six to eight weeks in *E. nitens*, Moncur et al. 1994) and even earlier at the molecular level. In *E. globulus*, as in most eucalypts, during early flower development the sepal primordia fuse to each other and the petal primordia fuse to form an outer and inner operculum, respectively. The entire umbel is enclosed by a pair of bracts. These are shed early during development as

the bud elongates, followed by the shedding of the outer (sepaline) operculum. The shedding of the inner (petaline) operculum to expose the reproductive structures is the process commonly referred to by eucalypt researchers as "flowering". Here, we will use the term anthesis for the shedding of the inner operculum, to avoid confusion with the flower initiation stage, as the latter corresponds to the stage generally referred to as "flowering" in the model annual plant *Arabidopsis*.

Eucalypts are not mast flowering species; populations generally flower relatively regularly and the anthesis period can be long, though there may be year to year variation in abundance (House 1997). There is natural variation in anthesis time across the range of *E. globulus*, and common environment field trials have shown that this trait has a genetic basis with a difference in peak anthesis time of up to eight months among genotypes (Gore and Potts 1995). Surprisingly, however, trees from different races planted in a seed orchard near Hobart, Tasmania, do not appear to vary in the timing of macroscopic appearance of flower buds, which occurs in spring in all provenances. Therefore, early anthesis genotypes appear to have a shorter flower bud development time than later anthesis genotypes. While anthesis time appears to be under strong genetic control, there is, however, noticeable year-to-year variation in the onset and duration of anthesis, which indicates that variation in climate, rather than photoperiod, is likely to be a key driver of variation in anthesis time.

Temperature influences the developmental rate of many organisms, especially plants, where a certain amount of heat is required before a particular developmental process occurs. The measure of accumulated heat above a base temperature is known as heat sum, or thermal time, and is measured in degree days or degree hours. Heat sum has been implicated in the timing of many developmental processes, especially in agricultural crops, and also trees for fruit production and forestry (reviewed in Trudgill et al. 2005). In trees, heat sum models have been used to analyse the effects of temperature on developmental processes such as spring bud burst (emergence of new leaves) in temperate and boreal trees, and seasonal anthesis time. For example, most of the year-to-year variation in peak pollen shed by *Pinus elliottii*, *P. palustris*, *P. taeda* and *P. echinata* can be attributed to heat sum (Boyer 1978). Using 19 years of flowering observations for *P. palustris* and six years of data for the other species, the average deviation from observed to predicted (based on heat sum) peak anthesis was four days or less (Boyer 1978). Heat sum models have also been used to predict anthesis time in

other trees such as almond (Rattigan and Hill 1986, 1988), Norway spruce (Nikkanen 2001), *Quercus* (Garcia-Mozo et al. 2006; Rodriguez-Rajo et al. 2005), *Alnus glutinosa* (Gonzalez-Parrado et al. 2006), *Prunus padus* and *Tilia cordata* (Thompson and Clark 2006), olive (Perez-Lopez et al. 2008) and nine shrubs and trees in the Mediterranean (Spano et al. 1999). In some cases heat sum alone accounted for the year-to-year or site-to-site variation in anthesis time, but in other cases a model combining chilling units and heat sum was used.

There are indications that heat sum may play a role in the year-to-year or site-to-site variation in annual anthesis time in eucalypts. For example, the anthesis time of the exotic *Eucalyptus nitens* in Tasmania was earliest in years that had the greatest annual heat sum (Barbour et al. 2006). Anthesis was also later in high altitude compared to low altitude plantations of unknown provenance, and the anthesis period was also shorter at high altitudes (Barbour et al. 2006; Moncur et al. 1994). Earlier anthesis at low altitudes has also been observed in other eucalypts (Pryor 1976). For example, in natural stands of *E. regnans*, anthesis commenced approximately two weeks earlier at lower altitudes than at high altitudes (Ashton 1975; Griffin 1980). Temperature has also been implicated in the site-to-site variation observed in honey-producing eucalypts in south-eastern Australia, as northern (usually warmer) sites often flower earlier than southern sites (Birtchnell and Gibson 2006).

The objectives of the present study were to determine the genetic and environmental control of flower initiation and flower opening time, including an investigation of the role of heat sum in controlling anthesis time in *E. globulus*.

MATERIALS AND METHODS

Seed orchard design

We studied trees growing in a common environment (seedEnergy Pty. Ltd. clonal seed orchard located at Cambridge, Tasmania: 42˚48’26”S, 147˚25’52”E; 40m elevation). All genotypes were selected based on superior breeding value for pulpwood production from the Southern Tree Breeding Association (STBA) breeding population. Six of the races defined by Dutkowski and Potts (1999) were represented in this orchard: Eastern Otways, Western

Otways, Strzelecki Ranges, Furneaux Group, South-Eastern Tasmania and Southern Tasmania (Fig. 1). Due to small sample size in some races, and as contiguous races have low differentiation at the molecular level (Steane et al. 2006), these races were pooled into four regions: Tasmania (South-Eastern Tasmania and Southern Tasmania), Otways (Eastern Otways and Western Otways), Strzelecki and Furneaux. Clones (i.e. ramets of each genotype) had been grafted onto *E. globulus* seedlings before planting, and later treated with paclobutrazol, a GA inhibitor that reduces internode length and increases the intensity of, but does not affect the timing of, flower initiation in eucalypts (Hasan and Reid 1995; Hetherington et al. 1992). *E. globulus* is a forest tree reaching heights of up to 90m in nature, but in the orchard, with grafting and treatment with paclobutrazol, trees were kept under five metres in height. As the orchard is used for mass supplementary pollination (Patterson et al. 2004), genotypes were arranged in multi-ramet line plots which varied in size from one to 18 ramets per plot. Across all years, sixty-three genotypes were assessed, but the sample size varied depending on the trait and season (Table 1). There were one to 15 plots per genotype. Nineteen genotypes were only represented by one plot, but as most of the analyses were conducted at the region level, these genotypes were included to improve the genetic representation. Plots and genotypes were effectively randomly distributed throughout the orchard.

Flowering surveys

Surveys were undertaken at least every two weeks during the flowering season of *E. globulus*, which generally lasts from May to February at the Cambridge seed orchard. Anthesis time was assessed by recording the percentage of unopened flowers on the trees at each survey (p), with the percentage decreasing from 100% to 0% during the time of scoring. Flower initiation was recorded as the survey date (number of days after 1 April each year) at which flower buds were first macroscopically visible, though the flower buds probably initiated at the microscopic level several weeks before they were visible to the naked eye. Anthesis time was scored in the flowering seasons starting in 2004, 2005, 2006, 2007 and 2008, but anthesis time was not surveyed for the entire length of the 2005 and 2006 seasons as the surveys in these seasons were conducted as part of the orchard's operational program. In 2008, daily survey data was available for some clones which were used to complete the estimation of anthesis time for that year. Flower initiation was scored in the 2006 and 2007 seasons. The

date of vegetative flush (Veg, the date at which new shoots appeared at the end of branches) was only recorded in 2007.

The anthesis time data were converted into three components for each tree (all measured in days from 1 April of each year): day at which anthesis commenced (AT1), day at which anthesis ended and peak anthesis time (ATpeak). Peak anthesis time is defined as the median anthesis time estimated from the scale and shape parameters derived by fitting a two-parameter Weibull function to the anthesis observational data expressed as (1-p/100) using PROC NLIN in SAS (SAS Institute; Version 9.1). This function was fitted separately for each tree in each year. Anthesis period (i.e. day at which anthesis ended minus AT1) was also calculated for each tree each year. Two different measures of flower initiation time were used, all measured from 1 April each year: (1) the day at which flowers were first macroscopically visible (FI, only measured in 2006 and 2007); and (2) estimated day of flower initiation, based on the region means averaged across 2006 and 2007 (FIregion, see Results section: Tasmania = 232, Furneaux = 230, Otways = 228, Strzelecki = 220).

Flower development

To compare flower development of an early and late anthesis genotype, several floral attributes were monitored on two ramets of a late anthesis genotype and one ramet of an early anthesis genotype. On each ramet, four branches with at least five flower buds each were tagged and measured every two weeks from 17th January 2007 until time of anthesis. The branches faced due north, south, east and west. Flower bud length and width were measured; and the timing of the shedding of the flower bract and inner and outer operculae were monitored.

Climatic data

Climatic data from the Hobart airport weather station (42˚49’59.16”S 147˚30’11.88”E, elevation 3m), which is only 6.5 km from the seed orchard and at a similar elevation, were provided by the Bureau of Meteorology, Hobart, for the period from October 2002 to February 2009. Mean daily temperatures were derived based on the average of the maximum and minimum daily temperatures. Heat sum, measured in degree days, is the linear

accumulation of temperature above a base temperature (Trudgill et al. 2005). In this study, five degrees was used as a base temperature as this was identified as the base temperature in a study of *E. globulus* growth (Reed et al. 2003). For each ramet, the period from FIregion to ATpeak was calculated each year for five years, and positive daily heat sum values (mean daily temperature minus five degrees) over this period were summed to determine "Heatsum" for each year.

Data analysis

Variation in each trait was analysed separately for each year as a mixed model using PROC MIXED in SAS (SAS Institute; Version 9.1), with region as a fixed effect, and genotype within region and plot within genotype as the random effects. Family effects were rarely significant as there was a low level of replication of genotypes within families so this level of hierarchy was excluded. When region effects were significant, least-square means were compared using Tukey-Kramer tests. A combined across-season analysis was undertaken using a repeated measures model with ramet within plot as the subject. In this case, the terms fitted were year, region, year x region (fixed effects) and genotype within region, plot within genotype, year x genotype within region, year x plot within genotype, and ramet within plot (random effects). The percentage of the total variance attributable to each term was estimated from variance components calculated treating all terms as random. These combined analyses including region were restricted to the three experimental assessments undertaken in the 2004, 2007 and 2008 seasons when all flowering genotypes were assessed.

The within-year broad-sense heritability at this site ($\hat{H}^2$) and its standard error were calculated for FI, ATpeak and anthesis period, using the program ASReml (Gilmour et al. 2006). The model fitted year (all five assessment years) as a fixed effect and genotype, plot within genotype and their interactions with year, and ramet within plot as random effects. The model ignored region of origin and thus the genotype term included region and within region genetic effects. The broad-sense heritability (Falconer and MacKay 1996) was calculated by dividing the genotype variance component by the sum of all variance components for all random terms in the model, including a pooled residual term. This estimate may be biased downward due to the relatedness of genotypes from the same family and these broad-sense heritabilities are presented here for comparison across traits and to provide an indication of

the repeatability of the traits. Correlation analyses were used to study the associations between traits, using PROC CORR in SAS (SAS Institute; Version 9.1). Spearman rank correlations (ρ) were calculated at the phenotypic (i.e. individual trees ignoring genotype in the analysis, ρ_p) and genotypic (genotype arithmetic means, ρ_g) levels for FI in 2006 and 2007, FI and Veg in 2007, Veg in 2007 and AT1 in 2008, AT1 and ATpeak within each year, FI 2006 and ATpeak 2007, FI 2007 and ATpeak 2008, and ATpeak among all years. As the results were effectively the same for ρ_p and ρ_g, only results for ρ_g were shown. A subset of individual trees that had a complete five year series of ATpeak records (n = 70) was selected to study the differences among years in Heatsum. The difference in heat sum between years was studied by fitting a one way repeated measures model with year as a fixed effect and differences between least squared means determined following a Tukey-Kramer adjustment.

RESULTS

Flower initiation and vegetative flush

In the orchard, flower initiation occurred nearly simultaneously across all trees. In 2006 the region and genotype within region effects were not significant ($F_{3,52}$ = 2.1, P = 0.115; Z = 1.2, P = 0.119), but in 2007 the effects were slightly significant ($F_{3,54}$ = 3.0, P = 0.04; Z = 2.3, P = 0.010). Flower initiation in 2006 and 2007 was not significantly correlated at the genotype mean level (ρ_g = 0.25, P = 0.06, n = 56). The genotype mean Spearman correlations between the timing of flower initiation and vegetative flush (i.e. FI and Veg) in 2007 were positive and significantly greater than zero (ρ_g = 0.57, P = 0.001, n = 32) and the mean time of vegetative flush was around two days earlier than mean flower initiation time in that year [mean Veg = 215 days (2 November); mean FI = 217 days (4 November)]. The timing of peak anthesis (ATpeak) in 2008 was not correlated with the timing of the vegetative flush in 2007 (ρ_g = 0.01, ns, n = 31). Flower initiation across the two years had a very low broad-sense heritability ($\hat{H}^2$) of 0.06±0.05.

There was a highly significant difference in mean flower initiation between the two years and a significant ($P < 0.05$) year x genotype within region interaction (Table 2). While the mean flower initiation was later in 2006 [mean = 239 (26 November)] than in 2007 [mean = 215 (2 November)], this may have been an artefact of scorer inexperience in detecting new flowers in the first year of the surveys, as the mode was 220 (7 November) in both years (Fig. 1). In the combined analysis the region effect was significant ($F_{3,52} = 3.71$, $P = 0.02$, Table 2), as the Strzelecki region had slightly earlier flower initiation time [region means averaged across 2006 and 2007: Tasmania = 232 (19 November), Furneaux = 230 (17 November), Otways = 228 (15 November), Strzelecki = 220 (7 November)]. There was no significant region by year interaction or overall genotype within region effect.

Timing of anthesis

The two measures of anthesis time (AT1 and ATpeak) were highly correlated each year (mean $\rho_g = 0.92$, ρ_g range 0.80-0.98, n = 5) and significantly greater than zero (all $P < 0.001$). As survey dates were sometimes up to two weeks apart, the calculated ATpeak was potentially more accurate than AT1 and therefore ATpeak was used in further analyses. The correlation between flower initiation time (FI) and peak anthesis time (ATpeak) were weak. The timing of anthesis in 2007 (ATpeak 2007) was not correlated with the time that those buds had initiated in the previous year (FI 2006) (Spearman $\rho_g = -0.14$, ns, n = 55). Similarly, there was no correlation between FI (2007) and ATpeak (2008) ($\rho_g = 0.13$, ns, n = 56).

In every year we analysed (five years) there was a significant difference (all $P \leq 0.05$) in peak anthesis time among regions and among genotypes within regions (e.g. Fig. 2). At the genotype mean level, rank ATpeak was highly positively correlated across years (mean ρ_g = 0.86, range 0.73-0.93, n = 10). This was also evident at the region level with anthesis occurring earliest in the Furneaux and Tasmania regions and latest in the Otways and Strzelecki regions each year (see Fig. 2 for 2004, 2007 and 2008 data). The tendency of the Strzelecki region to initiate flowers slightly earlier than other regions cannot account for the variation in anthesis time, as the Strzelecki region flowers late, not early (Fig. 2). There was also a high level of variation in the ATpeak trait among genotypes within regions (Table 2). For example, every year there was no overlap in anthesis period of two genotypes from

different families from the same locality (Parker Spur) in the Otways region, suggesting that they are reproductively isolated (Fig. 3).

In contrast to flower initiation time, peak anthesis time (ATpeak) in this orchard was highly heritable within years ($\hat{H}^2$ = 0.78±0.04). Anthesis period was under weaker genetic control ($\hat{H}^2$ = 0.09±0.05). Even including the year to year variation and the interaction terms, the majority of the phenotypic variation in ATpeak was attributable to genetic variation i.e. between regions (51%) and between genotypes within regions (29%) (Table 2). Year was one of the largest environmental effects (3%), and year x genotype within region and year x region interactions were significant but small compared with the main effects (Table 2).

Association between climatic variables and flower development

In the subset of samples for which there was a complete five year series of ATpeak records (n = 70), there was no significant difference in mean Heatsum among years ($F_{4,69}$ = 2.2, ns, Fig. 4a). This is despite there being a difference in mean ATpeak among most years for the same subset of trees ($F_{4,69}$ = 141.2, P < 0.001, Fig. 4b). For example, while anthesis occurred later in these trees in 2006 than in 2007 and 2008 (Fig. 4b), the average accumulated heat sum was not significantly different in these three years, as the same heat sum was accumulated earlier in the year in 2007 and 2008 than in 2006 (Fig. 4a), consistent with the cooler autumn in 2006. This provides good evidence that, overall, heat sum is a major driver of the year-to-year variation in peak anthesis time observed in this orchard.

Flower development

As flower bud initiation occurs at around the same time in the early and late anthesis genotypes, but there is variation in anthesis time, the difference in anthesis time is therefore mainly due to slower bud development in the late anthesis genotype. To investigate whether this difference was due to overall slower rates of bud development in the late anthesis genotype, or whether buds develop at the same rate but late anthesis genotypes sit dormant until anthesis time, bud growth was monitored in the early and late genotypes in the flowering season beginning in 2006. Buds were initiated on both early and late trees in spring, but flower bud development (macroscopic initiation until flower opening) lasted 26

weeks in the early ramet, and 54 weeks in the late ramets. The buds of both genotypes grew at a similar rate until the beginning of autumn (Fig. 5). Once the buds were at 80% of their final width, the buds of the early genotype continued to grow and opened in late autumn, but the buds of the late genotype entered a period of near dormancy which lasted until the following spring, after which there was a final growth spurt before opening (Fig. 5). The outer (sepaline) operculum was also shed earlier in development in buds of the early genotype than in those of the late genotype (Fig. 5).

DISCUSSION

The variation in the timing of anthesis in *E. globulus* is clearly under strong genetic control. Each year there was a significant difference among regions in the timing of anthesis, with Furneaux and Tasmania regions consistently earliest, and Strzelecki and Otways regions latest. There was, however, a significant year effect, which indicates that anthesis time is not a photoperiod-mediated response in *E. globulus*. Though the onset of anthesis in the orchard varied among years, the rank order of anthesis at the tree/genotype level was similar among years (i.e. Spearman's rank correlation was high). Instability in the anthesis time overlap of different species of eucalypt has been reported in long term flowering data sets (Birtchnell and Gibson 2006) but in the present case, the genetic-based barrier to gene flow between early and late anthesis genotypes of *E. globulus* appears relatively stable. The non-synchronous anthesis, does, however, have implications for management of seed orchards, as synchronous anthesis is important to optimise outcrossing of open pollinated seed. Non-synchronous anthesis is also considered a major problem in conifer seed orchards (Nikkanen 2001) although the reported anthesis periods of up to 30 days for conifer seed orchards (El-Kassaby and Askew 1991; El-Kassaby et al. 1984; El-Kassaby and Reynolds 1990) are much shorter than is observed in the Cambridge *E. globulus* orchard. Conversely, in *E. globulus* plantations, asynchronous anthesis of plantations and native stands would be desirable to minimise the rate of gene flow into native stands (Barbour et al. 2008). For example, in recent years, germplasm from *E. globulus* provenances from mainland Australia (Otways and Strzelecki) or Furneaux has been established in Tasmanian plantations, and some Tasmanian germplasm has been established near native stands on mainland Australia. Mainland Australian races are differentiated from Tasmanian races in microsatellite frequencies and numerous quantitative traits (Steane et al. 2006) and non-synchronous anthesis between some

plantations and native races will act as a pre-mating barrier to gene flow. By contrast, there does not appear to be a pre-mating barrier to gene flow between the Furneaux and Southern Tasmania regions but these two provenances are relatively closely related (Steane et al. 2006), which reduces the potential impact of gene flow.

There was very little genetic based variation in flower initiation time within the seed orchard (FI, $\hat{H}^2 = 0.06$). However, anthesis time was highly heritable in the broad-sense (ATpeak, $\hat{H}^2 = 0.78$), with approximately equal variation attributed to regions or genotype within regions. This high heritability occurred despite significant differences in anthesis time across years. Flowering time traits are often highly heritable, for example days to first flowering in annuals (e.g. cowpea $\hat{H}^2 = 0.95$, Machado et al. 2008; and lentil $\hat{H}^2 = 0.94$, Bicer and Sakar 2008) and seasonal anthesis in perennial species [e.g. days to peak flowering in almond narrow sense heritability (h^2) = 0.99, Dicenta et al. 1993; days to receptivity in black pine $\hat{H}^2 = 0.70$, Matziris 1994; days to peak anthesis in pistachio $h^2 = 0.79$-0.89, based on half-sib and parent-offspring regression analysis respectively, Chao and Parfitt 2003; and days to peak anthesis in kiwifruit $h^2 > 0.85$, Cheng et al. 2006]. A previous study of the genetic control of anthesis time in *E. globulus* calculated similarly high heritabilities of anthesis time traits for a single year ($h^2 = 0.65$, Gore and Potts 1995) and intermediate inheritance of racial differences in anthesis time in inter-race hybrids (Gore and Potts 1995), suggesting most of the observed genetic variation amongst genotypes and regions was due to additive genetic effects. In contrast to anthesis time, anthesis duration in perennials is under weak or variable genetic control (Cheng et al. 2006; Dicenta et al. 1993), a pattern that was also observed in this study ($\hat{H}^2 = 0.09$). This may be partly because the anthesis period is positively correlated with flower abundance (data not shown), which has a lower heritability (McGowen 2007).

While the variation in anthesis time in a common environment is under strong genetic control and thus relatively well predicted from pedigree information, there was still a significant environmental component, and year was one of the largest of the environmental effects. Various authors have suggested that heat sum may be involved in the year-to-year or site-to-site variation in annual anthesis time in eucalypts (Ashton 1975; Barbour et al. 2006; Birtchnell and Gibson 2006; Griffin 1980; Moncur et al. 1994; Pryor 1976). Indeed, when comparing trees for which there was a complete five year flowering record, the mean heat sum from flower initiation to anthesis for these trees was constant each year, which means

that the large between year variation in peak anthesis time of these trees of up to 30 days appear to have been caused by year to year fluctuation in temperature.

Many models of phenological development in trees incorporate chill units as well as heat sum, where a certain chilling requirement is necessary to break bud dormancy, followed by a heat sum requirement for flower development to anthesis. In almond, a combined chill unit:heat sum model based on data from one location could be used to predict anthesis time at a different location with a different climate (Rattigan and Hill 1988). Winter chilling has a role in flower bud initiation in *Eucalyptus nitens* (Gardner and Bertling 2005), and may also have a role in subsequent growth and development of flower buds in *E. globulus*. Measurements taken on developing flower buds showed that the differences in anthesis time were due to the late anthesis genotypes entering a period of near dormancy in flower growth while the flower buds of the early anthesis genotype continued to grow and open. This could indicate that the combination of chilling and heat sum requirement may differ from genotype to genotype. It may be that there is no chilling requirement in early anthesis genotypes, while in later anthesis genotypes a chilling period is required, and it is actually the heat sum accumulated after this chilling period that determines anthesis time. A model combining heat sum and chilling units would be worth testing. The role of heat sum in anthesis time has obvious implications for future management of *E. globulus* seed orchards, plantations and natural populations as there is clear evidence of global warming, including within the *E. globulus* range where the mean annual temperature is predicted to be 2-3˚C higher by 2100 (Christensen et al. 2007). Other studies have already shown that changes in phenology are occurring in response to shifting temperature regimes. For example, a meta-analysis of the phenology of 542 plant and 19 animal species over the last thirty years demonstrated a clear relationship between temperature and phenology, with evidence of advancing leaf unfolding, flowering and fruiting, and delays in leaf colouring and leaf fall in wild plants all across Europe (Menzel et al. 2006). On average, spring was advancing by 2.5 days per decade and leaf colouring and fall were delayed by 1.0 day per decade (Menzel et al. 2006).

E. globulus is a foundation tree species in lowland forests of south eastern Australia, and changes in anthesis time could have flow-on effects through the associated community as it could, in turn, affect the timing of nectar and pollen availability for birds and insects (Law et al. 2000). If changes in temperature affect the phenology of species differently, then the

synchrony in phenology between species could be disrupted. The swift parrot (*Lathamus discolor*), an endangered species, is an important pollinator of *E. globulus*. It feeds on the nectar and pollen of *E. globulus* flowers and the yearly reproductive success of the parrot is thought to depend upon the flowering intensity of *E. globulus* (Hingston et al. 2004). The relationship between *E. globulus* flowering and swift parrot migration could be altered if the increase in temperature affects the phenology of the two species differently. This is the case in oak where the moth egg hatch date has advanced more than the oak bud burst date over the last twenty years (van Asch et al. 2007). The high levels of genetic variation in anthesis time within and between populations could, however, give *E. globulus* the evolutionary flexibility to respond to future shifts in temperature and pollinator activity.

Within populations of *E. globulus* there is a large amount of genetic variation in anthesis time, to the point where different genotypes, even growing in close proximity, are effectively reproductively isolated. The maintenance of asynchronous anthesis within populations could be a means of reducing inbreeding, as pollinators are forced to move further during the flowering season (Elzinga et al. 2007), although asynchronous flowering could also increase inbreeding as the number of potential mates is reduced. *E. globulus* is both bird and insect pollinated, and the asynchronous anthesis may also help to avoid competition amongst trees for pollinators. Consistent with this hypothesis, a comparison of plants with wind and biotic pollination modes suggested that biotically pollinated plants had a wider phenological spread (Bolmgren et al. 2003), which could have evolved in the plants to prevent satiation of pollinators and/or increase their local persistence time.

In conclusion, this study has shown that anthesis time is controlled by mostly genetic but also environmental factors, both of which appear to be predictable to some extent. From a genetic perspective, there is a large amount of variation in peak anthesis time, with broad-scale differences among regions. From an environmental perspective, heat sum appears to be a significant factor in explaining at least the year to year variation in anthesis time, and is also likely to explain, at least in part, the differences in anthesis time that are observed among sites.

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ETHICAL STANDARDS STATEMENT

The experiments described here comply with the current Australian laws.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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FIGURES AND TABLES

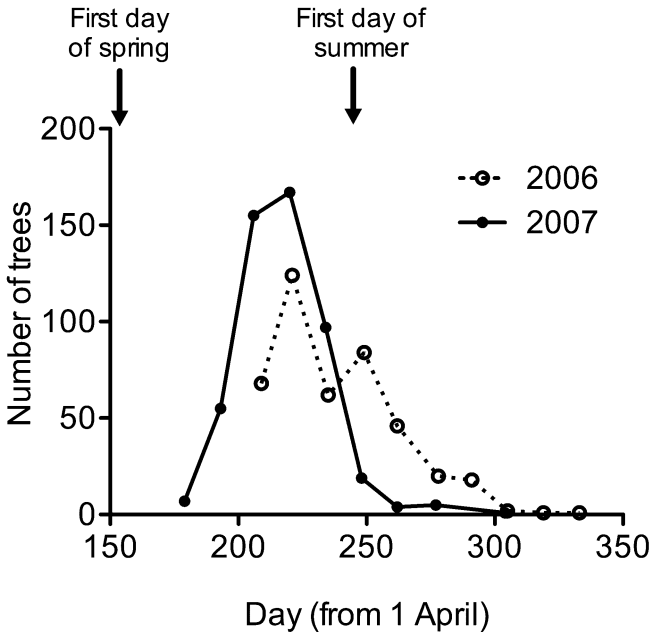

Fig. 1 Number of trees on which flower buds were observed for the first time at each survey day in 2006 and 2007 at the *E. globulus* seed orchard

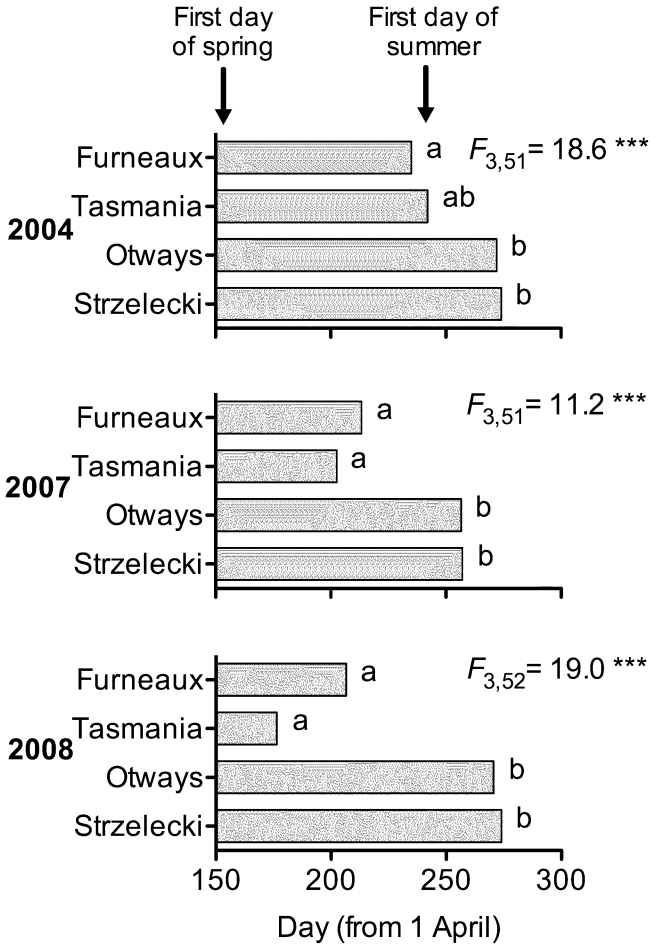

Fig. 2 Mean anthesis time (least-square mean ATpeak) for each region of *E. globulus* for three years (2004, 2007 and 2008) at the seed orchard. Region means sharing a letter within a year were not significantly different using the Tukey-Kramer test. ***, $P < 0.001$

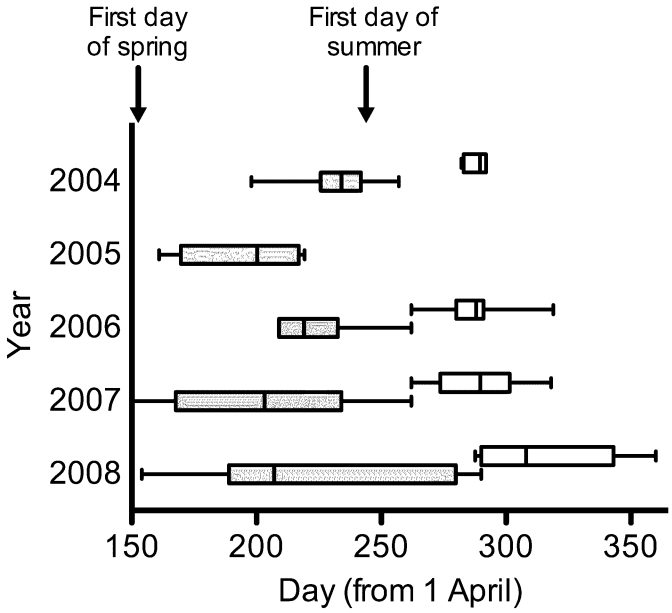

Fig. 3 Overall and peak anthesis period each year for two genotypes from the Parker Spur locality (Otways region of *E. globulus*). Box shows the range of calculated peak anthesis for ramets of each genotype (minimum and maximum ATpeak), with the genotype arithmetic mean ATpeak indicated by the line within the box; lines outside the box indicate the period of anthesis, from observed minimum AT1 to observed maximum day at which anthesis ended for each ramet of each genotype

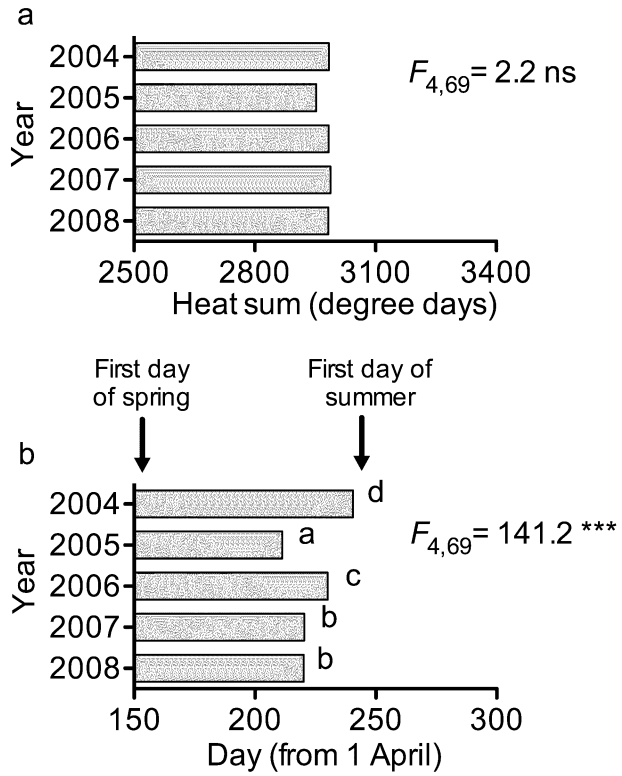

Fig. 4 Mean a) Heatsum and b) ATpeak each year in the 70 trees in the *E. globulus* seed orchard that had five complete years of ATpeak data. When the overall F test was significant, year means sharing a letter were not significantly different using the Tukey-Kramer test. ***, $P < 0.001$; ns, not significant

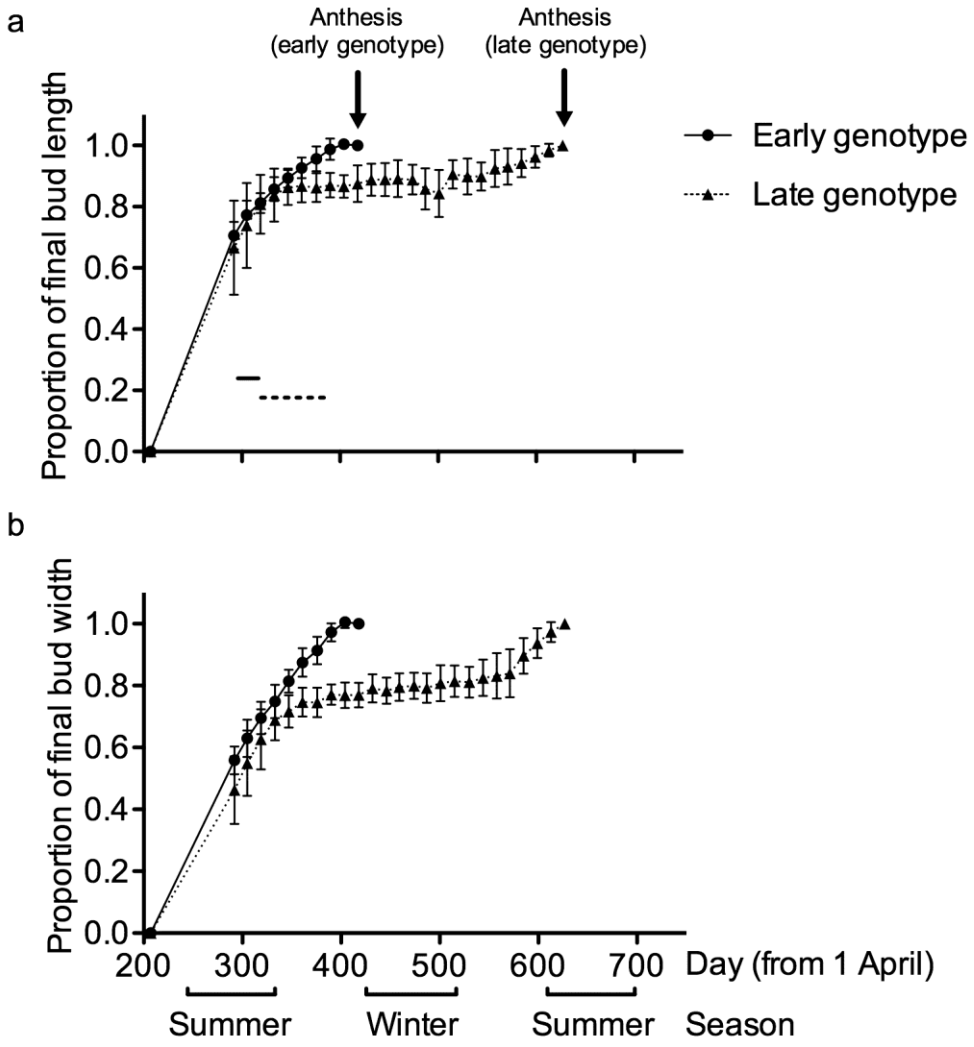

Fig. 5 Mean bud a) length and b) width of early and late anthesis genotypes of *E. globulus* at the *E. globulus* seed orchard. Error bars show standard deviation. Means are based on a sample size of 21 buds for the early genotype and 32 buds for the late genotype. The period of shedding of the outer (sepaline) operculum for each genotype is indicated on the top graph (solid line, early genotype; dashed line, late genotype). The time of shedding of the inner (petaline) operculum (anthesis time) of each genotype is also indicated. Note there were no measurements taken between initiation and week 34.

Table 1 Number (No.) of families, genotypes and individuals of *E. globulus* surveyed for flower initiation (FI) and peak anthesis time (ATpeak) from each region each year in the seed orchard. No. varies between years because not all individuals flowered each year.

		FI			ATpeak		
Year	Region	No. of families	No. of genotypes	No. of individuals	No. of families	No. of genotypes	No. of individuals
2004	Tasmania				3	3	23
2004	Furneaux				14	23	198
2004	Otways				8	14	84
2004	Strzelecki				12	15	89
2005	Tasmania				2	3	5
2005	Furneaux				9	15	81
2005	Otways				3	3	15
2005	Strzelecki				4	4	19
2006	Tasmania	3	5	22	3	4	20
2006	Furneaux	13	21	169	11	19	124
2006	Otways	9	16	102	8	15	97
2006	Strzelecki	12	14	82	11	14	82
2007	Tasmania	3	5	19	3	5	11
2007	Furneaux	14	23	211	13	21	173
2007	Otways	9	16	124	9	15	101
2007	Strzelecki	12	14	98	12	14	81
2008	Tasmania				3	5	18
2008	Furneaux				13	21	199
2008	Otways				9	16	123
2008	Strzelecki				12	14	94

Table 2 *F*-values (*F*), degrees of freedom (df) and significance (*P*) of fixed effects, and *Z*-values (*Z*) and significance (*P*) of random effects in the repeated measures model of flower bud initiation (FI) and peak anthesis time (ATpeak) for three years (2004, 2007 and 2008) at the *E. globulus* seed orchard. The proportion of phenotypic variation in ATpeak attributed to the different effects when all were treated as random is also shown, giving the percentage of variation due to genetic variation (among regions and genotypes within regions), interactive effects with year (year x genotype within region and year x region) and environmental variation (remaining components).

	FI		ATpeak		
Effect	*F* or Z (df)	*P*	*F* or Z (df)	*P*	Proportion of variation (%)
Fixed effects (*F* values)					
Year	89.9 (1,52)	< 0.001	19.8 (2,98)	< 0.001	3.0
Region	3.7 (3,52)	0.017	18.5 (3,56)	< 0.001	50.6
Year x region	1.1 (3,52)	0.376	4.9 (6,98)	< 0.001	2.3
Random effects (*Z* values)					
Genotype within region	0.5	0.324	4.7	< 0.001	28.5
Year x genotype within region	1.9	0.028	4.5	< 0.001	3.1
Plot within genotype	2.4	0.008	0.8	0.223	0.6
Ramet within plot	0.04	0.483	8.0	< 0.001	4.7
Residual					7.1